

The Exchange of Histidine C-2 Protons in Superoxide Dismutases

A NOVEL METHOD FOR ASSIGNING HISTIDINE-METAL LIGANDS IN PROTEINS

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The rates of exchange of the C-2 protons of histidine residues in copper-zinc superoxide dismutase are substantially decreased by metal ion binding. This observation was used to distinguish between ligand and non-ligand histidine residues in bovine and yeast copper-zinc superoxide dismutases; the effect was shown to depend only on metal ion co-ordination and not as a consequence of concomitant changes in protein structure. Selective deuteration of the zinc-only proteins at pH* (uncorrected pH-meter reading) 8.2 and 50°C resulted in the distinction between copper and zinc ligand resonances in the ^1H n.m.r. spectrum of the enzymes. This method is proposed as a generally applicable technique for identifying histidine residues as ligands in metalloproteins.

The physical, chemical and biological properties of metal centres in metalloproteins are mainly determined by the number, nature and geometry of those amino acid residues that act as ligands (Brill, 1977). Although the most unequivocal evidence of the nature of the metal-binding site in metalloproteins is provided by X-ray crystallography, many other, mainly spectroscopic, techniques have been used to elucidate the structures of these metal-binding sites (Brill, 1977). A major disadvantage of most spectroscopic methods used is that they are based on transitions centred on the metal ion rather than on the ligands. That is, deductions about the nature, number and orientation of the co-ordinating groups are based on the observation of optical or magnetic transitions associated principally with the metal ion, inasmuch as it can be considered separately from its ligands. It would seem preferable therefore to probe the nature of the metal ligands by the use of a 'ligand-centred' spectroscopic technique.

^1H n.m.r. spectroscopy is just such a technique. It is particularly useful in studying histidine residues, and several criteria have been shown to reflect the co-ordination of a metal ion (Cass *et al.*, 1977a; Baldwin *et al.*, 1978). These criteria include: the

change in pH-titration behaviour of the C-2 proton resonances in the holoprotein, as compared with the apoprotein (Cass *et al.*, 1977a; Baldwin *et al.*, 1978); a change in the chemical shift of the histidine C-2 proton resonances on binding metal ions (Baldwin *et al.*, 1978; Cass *et al.*, 1979a); the observation of nuclear spin-spin coupling between the metal-ion nucleus and histidine C-2 and C-4 protons (Cass *et al.*, 1978); change in the chemical shifts of C-2 proton resonances on addition of anions or molecules that act as ligands (Cass *et al.*, 1979b).

All but one of these criteria suffer from the criticism that they may be due to indirect effects of metal binding rather than to co-ordination itself. Only the observation of metal-proton heteronuclear spin-spin coupling is due to a direct through-bond metal-ligand interaction; however, this method is not widely applicable.

An alternative approach in studying metal co-ordination in proteins is to use chemical modification and to compare the behaviour of the apo- and holo-proteins. The principle of this method is that the metal ion will protect co-ordinating residues from reaction with the modifying reagent (Stokes *et al.*, 1974; Lippard *et al.*, 1977). However, chemical-modification studies are also subject to the indirect effects of the metal ion (Zisapel, 1978). In addition

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the modifying group is often bulky and may cause changes in the protein tertiary structure that make a comparison of native and modified proteins difficult.

In the present work we describe how a minimal chemical modification, the replacement of histidine C-2 protons by deuterons, studied by ^1H n.m.r. spectroscopy can be used to distinguish between zinc and copper ligands in bovine and yeast superoxide dismutases.

Materials and Methods

Enzymes

The copper-zinc superoxide dismutases from bovine erythrocytes (Bannister *et al.*, 1971) and baker's yeast (*Saccharomyces cerevisiae*) (Hasemann *et al.*, 1979) were isolated as described. Apoproteins were prepared by dialysis against EDTA as previously described for the bovine (Weser & Hartmann, 1971) and yeast (Cass *et al.*, 1979b) enzymes. In both cases the excess EDTA was removed by the method of Fee (1973). Reconstituted enzymes were prepared by adding a single stoichiometric quantity of zinc(II) sulphate (Hazelwood Chemicals, Staines, Middx., U.K.) solution to the apoprotein at pH 6.3 followed by incubation for 24 h at 4°C to give the zinc-only protein [two zinc(II) ions per 31200-dalton dimer]. A stoichiometric amount of a copper(II) sulphate (BDH, Poole, Dorset, U.K., AnalaR grade) solution was then added in five to ten portions, at 5 min intervals, to a solution of the two-zinc enzyme at pH 3.8 followed by incubation for 24 h at 4°C. After the pH had been adjusted to 6.5, this solution was freeze-dried. The enzyme reconstituted by this method gave a product ($\text{Cu}_2\text{-Zn}_2$ enzyme) with an ^1H n.m.r. spectrum identical with that of the native enzyme.

Deuteration procedures

Different enzyme samples were deuterated by the following procedure; 40–50 mg of the protein was dissolved in 7–10 ml of $^2\text{H}_2\text{O}$ (99.8% ^2H ; Merck, Sharpe and Dohme, Montreal, Que., Canada) containing 20 mM-sodium phosphate buffer and 0.02% (w/v) NaN_3 (to prevent bacterial growth) in sample tubes. The pH* (uncorrected pH-meter reading) of the solution was adjusted to 8.2–8.4, and the tubes were stoppered and incubated for 12 days at 50°C. At the end of the incubation the samples were dialysed against five changes of water, centrifuged to remove small amounts of precipitate and freeze-dried.

Sample preparation for n.m.r.

The deuterated enzymes were prepared for ^1H n.m.r. spectroscopy in $^2\text{H}_2\text{O}$ solutions containing either 1 M-NaCl (bovine) or 1 M- NaClO_4 (yeast) and 20 mM-sodium phosphate buffer in a total volume of 400 μl . The pH of the solutions in the n.m.r. tube was

measured with a Pye Ingold microelectrode and a Pye-Unicam PW 80 pH-meter. The pH* was adjusted with solutions of 100 mM- ^2HCl or - NaO^2H . The copper(II) enzyme was reduced by adding minimal solid sodium dithionite.

^1H n.m.r. spectroscopy

^1H n.m.r. spectra were obtained at 270 MHz in the Fourier-transform mode by using the Oxford Enzyme Group spectrometer consisting of a modified Bruker Console, a 6.4 T super-conducting magnet and BNC 12 computer. Routinely, 1024 transients were accumulated at a sampling frequency of 4000s^{-1} after a 70° pulse and with a pulse-to-pulse time of 0.6 s, with the use of quadrature phase detection. The excitation pulse was centred on the water frequency and the signal due to residual HO^2H suppressed by the application of a second pulse gated with respect to data acquisition (Campbell *et al.*, 1977). Solvent ^2H was employed as an internal field-frequency lock, and all chemical shifts are quoted in p.p.m. downfield from sodium 2,2-dimethyl-2-silapentane-5-sulphonate (DSS) as an internal standard.

To improve the resolution of the n.m.r. spectra the convolution-difference technique of Campbell *et al.* (1973) was employed.

Results

After incubation of bovine and yeast apo-(superoxide dismutases) at pH* 8.2–8.4 and 50°C for 12 days, the ^1H n.m.r. spectra show a complete loss in intensity of those resonances previously assigned to the C-2 protons on the basis of their pH*-titration behaviour (Cass *et al.*, 1977a, 1979b). Reconstitution and reduction of the deuterated apoprotein results in samples having the spectra shown in Fig. 1(a) (bovine) and 2(a) (yeast). ^1H n.m.r. spectra of the native proteins are shown in Figs. 1(d) (bovine) and 2(d) (yeast). The spectrum of the bovine enzyme after deuteration shows the loss of resonances 1–6 and 10; the yeast enzyme shows the loss of resonances 1–5 after deuteration.

Deuteration of the holoenzymes under the same conditions as used for the apoproteins shows only the loss of two (1 and 5) resonances in the bovine enzyme and of none in the yeast enzyme [Figs. 1(c) and 2(c) respectively]. After deuteration of the zinc-only proteins the ^1H n.m.r. spectra of the reconstituted samples show the selective loss of four resonances (1, 2, 4, and 5) in the bovine enzyme and two resonances (2 and 3) in the yeast enzyme. This is illustrated in Figs. 1(b) and 2(b) respectively.

In addition to the loss of intensity of the sharp C-2 proton resonances, these spectra also show variable changes in the intensity of the broad resonances of 8–10 p.p.m., assigned to the peptide backbone protons

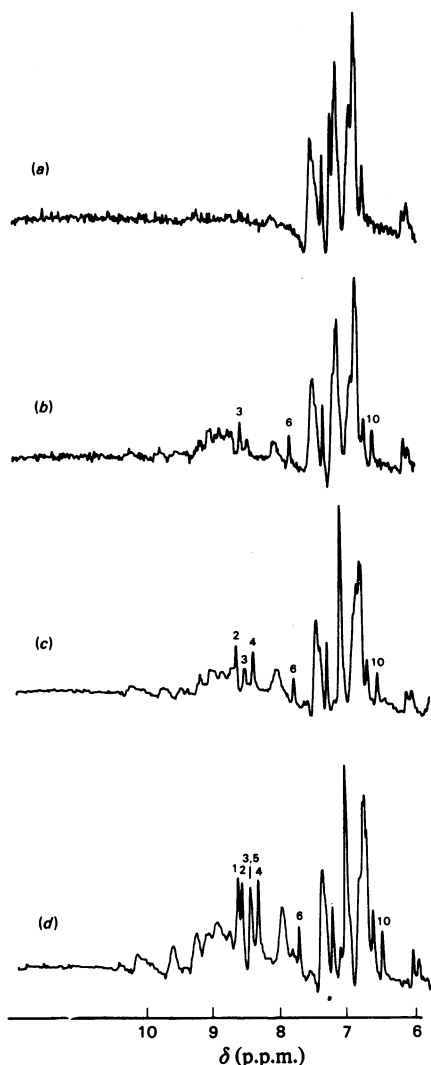


Fig. 1. Low-field region of 270 MHz ^1H n.m.r. convolution difference spectrum of copper(I)-zinc(II) bovine erythrocyte superoxide dismutase in $^2\text{H}_2\text{O}$ containing 1M-NaCl and 20mM-phosphate buffer at pH* approx. 6.5 and 40°C. Protein concentration was 80–100mg/ml. (a) Reconstituted enzyme after deuteration of the apoprotein; (b) reconstituted enzyme after deuteration of the zinc-only protein; (c) native enzyme after deuteration; (d) native enzyme.

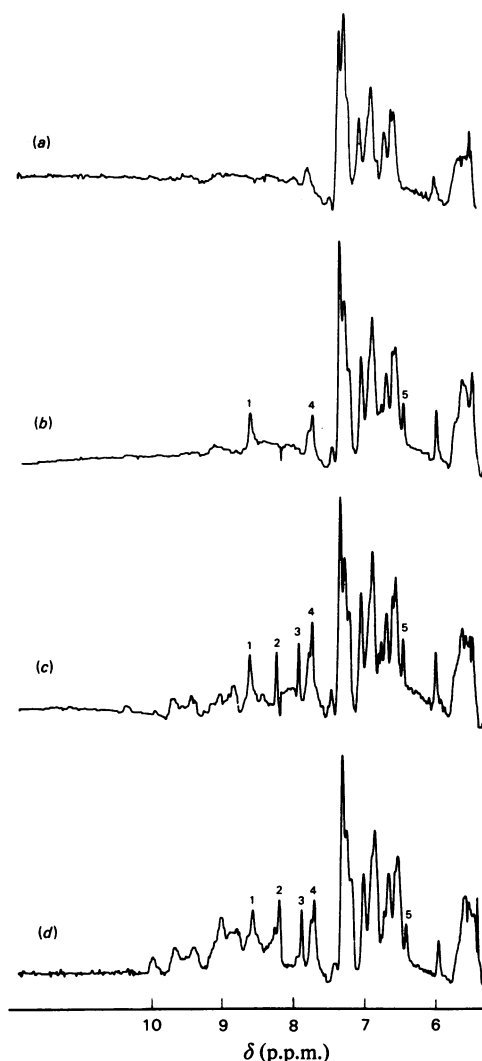


Fig. 2. Low-field region of 270 MHz ^1H n.m.r. convolution difference spectrum of copper(I)-zinc(II) yeast superoxide dismutase in $^2\text{H}_2\text{O}$ containing 1M- NaClO_4 and 20mM-phosphate buffer at pH* approx. 6.5 and 40°C. Protein concentration was 80–100mg/ml. (a) Reconstituted enzyme after deuteration of the apoprotein; (b) reconstituted enzyme after deuteration of zinc-only protein; (c) native enzyme after deuteration; (d) native enzyme.

(Campbell *et al.*, 1975). Intensity changes in this region reflect the change in exposure of the peptide backbone to the solvent (Campbell *et al.*, 1975). This is an indirect effect of metal co-ordination on the behaviour of the protein and shows that the apo-

enzyme has a more open structure than the holo-enzyme.

The attempted deuteration of the copper-only protein under the same conditions results in a non-selective partial intensity loss of all of the histidine C-2 proton resonances.

Discussion

A comparison of Figs. 1(a)–1(d) and of Figs. 2(a)–2(d) shows that the presence of bound metal ions substantially lowers the rate of deuteration at the C-2 position of histidine residues in both bovine and yeast superoxide dismutases.

Apoproteins

The ^1H n.m.r. spectra of the apoproteins of both bovine and yeast superoxide dismutases show resonances assigned to all eight (bovine; Steinman *et al.*, 1974) or all six (yeast; Overballe-Petersen *et al.*, 1979) histidine C-2 protons (Cass *et al.*, 1977a, 1979b). After incubation at pH* 8.2–8.4 for 12 days at 50°C none of these resonances can be observed in the ^1H n.m.r. spectra (not shown); this behaviour has been observed in several other proteins (Krieger *et al.*, 1976; Markley & Cheung, 1973), and, although exchange half-times were not measured, effectively complete exchange after 12 days is consistent with observations of other proteins.

The reconstitution of these proteins with their native metals shows that the resulting ^1H n.m.r. spectra have also lost several sharp resonances [Figs. 1(a) and 1(d), and 2(a) and 2(d)]. In the bovine enzyme the missing resonances correspond to those labelled 1–6 and 10 in the spectrum of the native protein (Fig. 1d), whereas in the yeast enzyme resonances 1–5 are not observed. Although in each protein the number of missing resonances is one less than the total number of histidine residues, the results described above for the apoproteins show that all of the histidine residues had been deuterated.

There are several reasons why one of the C-2 protons is not observed in the holoprotein; the resonances may be substantially upfield-shifted, and occur under the water peak, or it may have a shorter spin–spin relaxation time than expected and thus gives rise to a very broad resonance. Alternatively the peak may be shifted upfield into the main aromatic envelope, where it is not sufficiently well resolved from the other resonances in this region.

In the bovine enzyme, resonances 1–6 had been previously assigned to C-2 protons on the basis of their linewidths and chemical shifts (Cass *et al.*, 1977a) and the resonance labelled 10 had been assigned to a C-4 proton. The present deuteration results suggest that this peak should be reassigned to a C-2 proton. Exchange at the C-4 (C-5) position in imidazole and its derivatives only occurs under much more severe conditions than those for C-2 proton exchange (Vaughan *et al.*, 1970) and has never been reported in proteins.

Similarly the results with the superoxide dismutase from yeast implicate peaks 1–5 as C-2 proton resonances.

Holoproteins

The ^1H n.m.r. spectra of the holoproteins after deuteration show the loss of either two (bovine) or no (yeast) C-2 proton resonances. In the spectrum of the bovine enzyme the two missing resonances, 1 and 5 (Fig. 1c), were previously assigned to non-ligand histidine residues 41 and 19 respectively (Cass *et al.*, 1977a,b). The remaining resonances are from ligand C-2 protons and do not exchange under these conditions. A sequence comparison of the bovine (Steinman *et al.*, 1974) and yeast (Overballe-Petersen *et al.*, 1979) enzymes reveals that histidine residues 19 and 41 have been replaced by a lysine and an arginine residue respectively, and consistent with this is the absence of resonances corresponding to 1 and 5 from the ^1H n.m.r. spectrum of the yeast enzyme (Cass *et al.*, 1979b). Similarly to the bovine enzyme, the unchanged histidine C-2 protons arise from the conserved ligand residues.

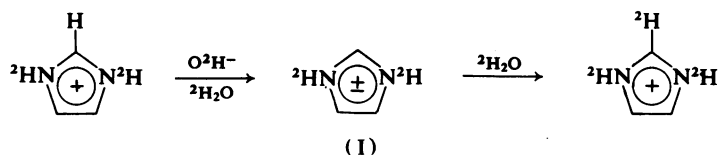
These results with both the bovine and yeast enzymes are completely consistent with the premise that only non-co-ordinated histidine residues undergo deuterium exchange at the C-2 position.

Zinc-only protein

The differential rate of deuteration in the apo- and holo-proteins described above could be due to an indirect effect of the metal binding. Indeed both the extent of exchange of the amide protons on the peptide backbone described above, and previous ^1H n.m.r. studies (Cass *et al.*, 1977a; Fee & Phillips, 1975; Lippard *et al.*, 1977) have shown a change in structure on metal ion binding. This structural change has been shown to be promoted by zinc(II) ion binding (Lippard *et al.*, 1977; Cass *et al.*, 1979a), and on the basis of ^1H n.m.r. spectra the holoproteins and zinc-only proteins have very similar structures (Lippard *et al.*, 1977; Cass *et al.*, 1979a).

Fig. 1(b) shows the ^1H n.m.r. spectrum of a reconstituted sample of bovine superoxide dismutase after deuteration of the zinc-only protein. In this case the spectrum shows the loss of four resonances, 1 and 5, due to non-ligands, and 2 and 4, which are from ligand histidine C-2 protons. Resonances 3, 6 and 10 do not lose intensity under these conditions.

A similar result is obtained with the yeast enzyme: resonances 2 and 3 disappear, whereas resonances 1, 4 and 5 are unaffected. These results are consistent with the effects of Cl^- on the ^1H n.m.r. spectra; Cl^- has been shown to bind only to the copper centre in the copper(I)–zinc(II) protein (Fee & Ward, 1976). When the yeast enzyme is titrated with Cl^- , resonances 2 and 3 show a large downfield shift, and resonance 5 shows a much smaller downfield shift (Cass *et al.*, 1979b). The bovine enzyme shows a similar large downfield shift of resonances 2 and 4 upon Cl^- titration, although in this case resonance 10



Scheme 1. *Proposed mechanism for the exchange of the C-2 proton in imidazole derivatives (Markley & Cheung, 1973; Vaughan et al., 1970)*

was not perturbed (A. E. G. Cass & H. A. O. Hill, unpublished work).

All of the above data are consistent with the suggestion that only non-co-ordinated histidine residues will undergo deuterium exchange at the C-2 position. Table 1 summarizes the histidine assignments in both the bovine and yeast superoxide dismutases based on this work.

Copper-only protein

It was not possible to find conditions for selective deuteration of the copper-only protein. It appears that in the absence of a bound zinc(II) ion the copper(II) ion is only weakly or non-specifically co-ordinated to the protein; this would be consistent with the proposed role of the zinc(II) ion in pre-forming the copper-binding site as mentioned above.

Mechanism of exchange

The mechanism by which the co-ordination of a metal ion prevents the deuteration at the C-2 position of histidine has not been investigated. In imidazole and its derivatives the postulated mechanism of C-2

proton exchange is via the rate-limiting formation of an ylide intermediate (I) generated by abstraction of the C-2 proton (Vaughan *et al.*, 1970; Markley & Cheung, 1973) as shown in Scheme 1.

Factors that stabilize the intermediate will enhance the rate of deuteration, and the relative deuteration rates of various nitrogen heterocycles have been correlated with the calculated anion or ylide stabilities (Vaughan *et al.*, 1970). In proteins the deuteration of histidine residues is via the equilibrium concentration of the imidazolium form. Replacement of $^2\text{H}^+$ by M^{2+} (bivalent metal ion) lowers the rate of deuteration, and if the mechanism described above is still applicable, it does so by decreasing the stability of the intermediate that corresponds to (I). This decreased stability may be because appreciable back-donation of electron density from the metal on to the histidine ring lowers the net positive charge.

Since the amino acid sequences of both yeast and bovine superoxide dismutase are known, it should be possible to use tritiation combined with peptide 'mapping' to locate those histidine residues in the sequences that are ligands to the metal ions. Such an experiment would also show unambiguously that proton exchange at C-2 occurs only on non-co-ordinated histidine residues. This method has been used with the zinc metalloenzyme β -lactamase II (Baldwin *et al.*, 1979) and to identify the fast-exchanging histidine (41) in bovine superoxide dismutase (Cass *et al.*, 1977a).

Table 1. *Summary of C-2 proton assignments in bovine and yeast copper-zinc superoxide dismutases*

Classification of C-2 proton resonances in bovine and yeast superoxide dismutases is based on the results of selective deuteration experiments.

Bovine

Resonance	Assignment
1	Histidine-41
2	Copper ligand
3	Zinc ligand
4	Copper ligand
5	Histidine-19
6	Zinc ligand
10	Zinc ligand

Yeast

Resonance	Assignment
1	Zinc ligand
2	Copper ligand
3	Copper ligand
4	Zinc ligand
5	Zinc ligand

Conclusions

Comparison of the deuteration of histidine residues in apo-, zinc-only and holo-(superoxide dismutases) from yeast and bovine erythrocytes has shown that only those histidine residues not co-ordinated to metal ions will be deuterated readily at the C-2 position. This has enabled us to distinguish the histidine residues as ligands or non-ligands, and to determine the minimum number of each. Ligand histidine residues can be further distinguished as either zinc or copper ligands and again the minimum number of each can be determined. The identification of ligands by this method has been shown to be consistent with previous results based on different criteria for both the bovine and yeast enzymes.

These results lead us to conclude that the deuteration behaviour is only dependent on co-ordination to metal ions, and not on concomitant changes in structure, in superoxide dismutases. We suggest that this method and the analogous tritiation experiments provide a general method for studying histidine co-ordination in metalloproteins.

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